

Mitogenic responses of hamsters infected with *Treponema pertenu*

Lack of correlation with passive transfer of resistance

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SUMMARY Infection of the CB/Ss Lak hamster with *Treponema pertenu* is characterised by chronic cutaneous lesions and lymph nodes teeming with treponemes. Throughout the course of infection lymph node and spleen cells responded poorly to the mitogens concanavalin A, phytohaemagglutinin, and lipopolysaccharide. This impairment preceded clinical signs of infection and correlated well with the chronicity of framboesial infection. High concentrations of antigen from *T pertenu*, but not from the non-pathogenic *Treponema phagedenis*, depressed the mitogenic response of normal lymphoid cells. After framboesial hamsters were treated with penicillin the mitogenic activities of their lymph node and spleen cells were similar to or slightly raised above those of controls. No significant differences were detected among recipients of framboesial immune cells with or without mitogenic activity. Recipients of immune lymph node and spleen cells from penicillin-treated or non-penicillin-treated animals had no cutaneous lesions 21 days after infection and had significantly lower lymph node weights and fewer treponemes per node than recipients of cells from normal penicillin-treated or non-penicillin-treated animals. Since lymphocyte transformation in vitro does not correlate with in-vivo treponemicidal activity, it is not a valid approach to assess the protective immune capacity of the framboesial host.

Introduction

Cells obtained from hamsters infected with *Treponema pertenu*, the aetiological agent of yaws or framboesia, can confer protection on normal recipients challenged with the same strain.¹ The protective activity of the immune cells resides largely within a population of cells sensitive to treatment with antithymocyte serum and complement, preferentially stimulated by concanavalin A (con A) and devoid of surface immunoglobulins.^{2,3} The donors of these immune T cells, however, exhibit cutaneous lesions that persist for six or seven months. When cured of their primary disease with penicillin, the framboesial hamsters are themselves resistant to a challenge infection.⁴

This inability of hamsters to eliminate *T pertenu* after several months of infection,^{4,5} even though their

cells can confer protection on recipients, is a paradox. The chronicity of framboesial infection suggests that in the host an effective cellular immune response is impaired. In the present study we therefore attempted to ascertain whether the inability of framboesial hamsters to eradicate the primary infection was reflected in alterations of their lymphocytes' responses to mitogens before and after treatment with a curative dose of penicillin. In addition, lymphoid cells from penicillin-treated and non-penicillin-treated framboesial hamsters were tested for their in-vitro responses to mitogens, and the results were correlated with the cells' ability to confer protection in vivo on normal recipients.

Materials and methods

ANIMALS

Inbred CB/Ss Lak hamsters were obtained from Charles River Breeding Laboratories (Wilmington, Massachusetts, USA). Hamsters weighing 80-100 g were housed six per cage at an ambient temperature of 18°C, a condition which facilitates the development of cutaneous lesions.⁶

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ORGANISM AND INFECTION

T. pertenu Haiti B was obtained from Dr Paul B Hardy jun (Johns Hopkins University, Baltimore, Md, USA) and was maintained by passage in hamsters. The inguinal lymph nodes were removed aseptically 3-4 weeks after intradermal infection, teased apart in sterile saline or tissue culture medium (RPMI 1640), and filtered through 60-mesh stainless-steel screens to achieve a single-cell suspension. After centrifugation at $270 \times g$ for 10 minutes to remove cellular debris the number of treponemes in the supernatant was determined by darkfield microscopy.

Experimental groups of hamsters were infected intradermally in the inguinal area with 1×10^6 *T. pertenu* suspended in saline. Cutaneous lesions developed within 2-3 weeks in all hamsters inoculated with 10^6 organisms. Changes in the weight of the inguinal lymph nodes and the number of treponemes in the nodes were also used to evaluate the host response to infection.⁴ The same criteria were used to evaluate the immune response of hamsters adoptively immunised with cells from framboesial donors.

PREPARATION OF SPLEEN, LYMPH NODES, AND BONE MARROW CELLS

Spleen and lymph nodes were removed aseptically, placed in RPMI 1640 medium, teased apart with forceps, and pressed through a 60-mesh stainless-steel screen to achieve a single-cell suspension. Each suspension was washed three times in RPMI 1640 with 10% fetal calf serum (FCS) at $210 \times g$. Bone marrow cells were flushed from the femur and humerus with RPMI 1640 medium and an 18-gauge needle and syringe. Clumps of bone marrow were dispelled by aspiration and expulsion through the needle. The number and viability of white cells were determined by the trypan blue exclusion test.

MITOGEN STUDIES

Single-cell suspensions of spleen and lymph node cells from normal and infected animals were prepared and diluted to the proper cell concentrations in RPMI 1640 with 10% heat-inactivated FCS containing 100 units penicillin and 100 µg streptomycin per ml (Gibco). Suspensions of 0.1 ml of medium containing 5×10^5 spleen or 1×10^6 lymph node cells were cultured in flat-bottomed 96-well microtitre dishes (Costar, Cambridge, Mass, USA) at a final volume of 0.2 ml per well.

Three mitogens, con A (Sigma), lipopolysaccharide from *Escherichia coli* 055:B5 (LPS; Difco), and PHA (Difco), were prepared according to the manufacturers' instructions and diluted in RPMI 1640

medium containing 10% FCS. They were added to cell cultures in the following concentrations: con A, 0.1 - 10 µg/ml; LPS, 1 - 100 µg/ml; and PHA, 10 , 20 , or 40 µl undiluted. The optimal amounts per culture were: con A, 1 µg; LPS, 100 µg; and PHA, 20 µl.

Cultures were incubated in duplicate at 37°C for 72 hours in hermetically sealed, humidified CO_2 culture boxes with a mixture of 5% CO_2 and 95% air. Twenty-four hours before harvesting each culture was pulsed with 1 µCi of ^3H thymidine (specific activity 6.7 Ci/mole, New England Nuclear). Cultures were harvested with a Skatron multiple-cell-culture harvester (Flow Laboratories) on to filter paper. The dried filter-paper discs were placed in Scintosol (Isolab) and counted in a Packard scintillation counter.

Mean \log_{10} counts/min were determined for both the stimulated and unstimulated subgroups of the infected and normal cultures. A stimulation index was then calculated as: infected (stimulated - unstimulated)/normal (stimulated - unstimulated).

A ratio of <1 was considered to indicate impairment of the infected lymphocytes' responsiveness to the mitogen.

PREPARATION OF TREPONEMAL ANTIGENS BY SONICATION

Four weeks after infection with 1×10^6 *T. pertenu* framboesial antigens were prepared by teasing apart the infected lymph nodes with forceps and gently pressing them through a 60-mesh stainless-steel screen into RPMI 1640 medium. The cellular debris was removed by centrifugation at $270 \times g$ twice for 10 minutes each. The supernatant containing treponemes was removed and washed four times with RPMI 1640 medium by centrifugation (Sorvall RC-2B) at $25\,000$ rev/min for 20 minutes. The treponemes were resuspended to 1×10^7 organisms/ml in saline and sonicated for 50 minutes at 0°C in an Artek sonic dismembrator at maximum power. Normal lymph node cells were extracted by the same procedure and served as a control for contamination by hamster cells.

For comparative study a non-pathogenic treponeme, *T. phagedenis* biotype Reiter, was cultured in spirolate broth at 37°C under anaerobic conditions. The organism was washed four times with saline and resuspended to 1×10^7 organisms/ml before sonication.

The sterility of the antigen preparations was tested by inoculating them on to various bacteriological media and incubating the cultures at 37°C for 48 hours. No contamination was observed. The amount of protein in the sonicated suspensions was determined by the method of Lowry *et al.*⁷

ADOPTIVE TRANSFER OF RESISTANCE

Thirty hamsters were infected intradermally with 1×10^6 *T. pertenuis* in the inguinal region. Nine weeks after inoculation half of these hamsters were treated with penicillin (4000 units) intramuscularly. Concurrently 10 of the normal group of hamsters were treated with penicillin. Ten days later all of these hamsters (infected and normal, penicillin-treated and non-penicillin-treated) were killed. Separate pools of spleen and lymph node cells were made for each of the four resulting groups.

A fresh group of normal hamsters was then inoculated intravenously with 7×10^7 viable cells each. Although all hamsters used in this study were inbred, the recipients were first irradiated as an additional precaution against a host-versus-graft reaction. For this purpose they were placed in Lucite containers and exposed to 900 rads of gamma-irradiation from a Gammator Cs-137 irradiator (Isomedix Inc). Irradiated hamsters lived only 9-14 days unless they were reconstituted with 6×10^7 bone marrow cells from normal syngeneic hamsters. Cells from the eight donor pools (see table III) were then transferred intravenously. After 24 hours the recipients and the remaining donor animals were infected intradermally with 1×10^6 *T. pertenuis*. Four weeks after infection the animals were killed and the extent of treponemal infection determined.

STATISTICAL ANALYSIS

The Fisher least-significant-difference test⁸ was used to examine pairs of means when a significant F ratio indicated reliable mean differences. The alpha level was set at 0.05 before the experiments started.

Results

INFECTION WITH *T. PERTENUIS* AND IN-VITRO LYMPHOID RESPONSES TO T-CELL AND B-CELL MITOGENS

A large group of hamsters was infected with 1×10^6 *T. pertenuis*. Approximately 19 ± 2 days after infection each site of inoculation became a large crusted lesion, and the periphery of the lesion continued to expand slowly for the next 16 weeks. From week 2 there was a rapid increase in the number of treponemes in the lymph nodes. The number peaked at six weeks (1×10^7 node) and then began to decrease, although 2×10^5 treponemes were still detected after 16 weeks of infection.

Each week for nine weeks and again at 16 weeks three framboesial and three control hamsters were killed and the responses of their lymph node and spleen cells to mitogens determined. Framboesial infection strongly inhibited the responses of lymph node cells to mitogens (table I). In general, the lymph node cells of framboesial animals responded poorly at all intervals to con A, LPS, and PHA. Although results for the optimal concentrations of the mitogens alone are shown in table I, similar results were obtained with suboptimum and supraoptimum concentrations. In contrast the response of framboesial spleen cells was variable, with periods of impairment and of enhancement to T-cell and B-cell mitogens. When these experiments were partially replicated similar results were obtained.

The same protocol was repeated with lymph node and spleen cells from hamsters that had been infected for 10 weeks and then treated with a curative dose of penicillin. Lymphocyte responses to each mitogen

TABLE I Relative mitogenic responses of normal and framboesial spleen and lymph node cells

Weeks after infection	Stimulation index*					
	Lymph node cells			Spleen cells		
	Con A	LPS	PHA	Con A	LPS	PHA
Untreated						
1	0.40	0.29	0.40	0.83	0.79	0.30
2	0.55	0.38	0.57	1.51	1.10	1.49
3	0.54	0.88	0.54	0.93	1.07	1.28
4	0.65	0.39	0.43	0.80	0.51	0.45
5	0.28	0.05	0.39	0.81	0.85	2.15
6	0.47	0.25	0.53	0.80	1.67	0.59
7	0.45	0.03	1.01	1.06	0.57	1.43
8	0.64	0.16	0.84	1.38	0.97	0.10
9	0.61	0.34	0.72	0.99	0.81	0.89
16	0.53	0.21	0.59	1.64	0.79	1.21
Penicillin-treated†	1.47	1.47	1.31	0.84	1.16	0.96

*Ratio of mean \log_{10} counts/min as follows: infected cells (stimulated-unstimulated)/normal (stimulated-unstimulated). A ratio of <1 indicated impairment of mitogenic activity. The values shown are for optimal concentrations of mitogens (per culture): con A, 1 μ g; LPS, 100 μ g; PHA, 20 μ l. Cultures were harvested after 72 hr of incubation. Each mean was an average of duplicate wells and three or more hamsters per determination.

†Framboesial hamsters were treated with penicillin after 10 weeks of infection. Response to mitogens was determined three weeks later.

were either close to or slightly higher than those of normal controls (table I). Similar results were obtained with framboesial hamsters cured 12 and 16 weeks after infection. These results suggested that impairment of lymphocyte reactivity is associated with the amount of treponemal antigen. To test that inference a series of experiments was performed.

IN-VITRO RESPONSES OF NORMAL LYMPHOID CELLS TO TREPONEMAL ANTIGENS

To confirm that the impaired reactivity of hamster lymph node and spleen cells was associated with the amount of treponemal antigen normal cells were exposed to the 3125-fold range of concentrations of sonicated *T. pertenu* or *T. phagedenis* in the presence or absence of PHA (table II). With high concentrations (5 µg and 25 µg/ml) of *T. pertenu* all responses were significantly impaired. No inhibition was observed when the lymphocytes were exposed to normal lymph node antigen or RPMI medium or when they were incubated with *T. phagedenis*.

After 48 hours in these cultures the number of viable cells was significantly reduced to 75% and 26% of controls (cells exposed to lymph node antigen or RPMI) in the presence of 5 µg and 25 µg *T. pertenu*/ml respectively. These results suggested that *T. pertenu* exerted a cytotoxic effect.

ABILITY TO CONFER RESISTANCE BY TRANSFER OF IMMUNE LYMPHOCYTES WITH OR WITHOUT MITOGENIC ACTIVITY

After 11 weeks of framboesial infection lymph node and spleen cells were obtained from infected

hamsters, penicillin-treated or non-penicillin-treated, and from the corresponding groups of normal hamsters. Their responses to mitogens were similar to those reported in table I. The remaining cells were transferred to irradiated, bone-marrow-reconstituted hamsters. Twenty-four hours later the recipients were infected intradermally with 1×10^6 *T. pertenu*. As additional controls, three surviving hamsters from each group of donors were similarly challenged. No new lesions developed in the framboesial donors, although lesions were detected in the normal donors (penicillin-treated and non-penicillin-treated) 19 days after infection.

On day 21 after infection all recipients were killed. In hamsters that had received immune cells from either penicillin-treated or non-penicillin-treated infected animals no cutaneous lesions were observed. The lymph nodes of these recipients weighed less and contained significantly fewer treponemes than lymph nodes from the recipients of cells from normal animals, whether penicillin-treated or not (table III). When this experiment was partially replicated, similar results were obtained.

Discussion

Framboesial infection of the hamster is characterised by chronic cutaneous lesions which persist for six or seven months, and by lymph nodes teeming with treponemes.⁴ The infected host is able to produce concomitantly humoral and cellular components that can confer a substantial degree of protection on recipients challenged with *T. pertenu*. When

TABLE II Effect of *T. pertenu* and *T. phagedenis* antigen on the response of normal lymphocytes to stimulation with PHA

Concentration of protein (µg)	Incorporation of (3H) thymidine (cpm)*			
	Lymph node cells		Spleen cells	
	PHA	None	PHA	None
<i>T. pertenu</i>				
0.008	4.50±0.03	3.79±0.04	4.33±0.06	3.95±0.02
0.04	4.47±0.03	3.70±0.01	4.33±0.02	3.92±0.01
0.2	4.33±0.12	3.67±0.01	4.36±0.02	3.90±0.01
1	4.40±0.03	3.65±0.07	4.15±0.04	3.79±0.01
5	3.45±0.03†	2.94±0.04†	3.14±0.01†	3.21±0.02†
25	2.64±0.05†	2.58±0.09†	2.55±0.06†	2.53±0.06†
Lymph node antigen	4.41±0.02	3.71±0.01	4.28±0.04	3.96±0.01
Control	4.37±0.01	3.62±0.05	4.29±0.06	3.84±0.04
<i>T. phagedenis</i>				
0.008	4.44±0.07	3.65±0.00	4.22±0.01	3.90±0.01
0.04	4.48±0.07	3.65±0.01	4.31±0.02	3.90±0.01
0.2	4.51±0.08	3.62±0.01	4.49±0.02	4.00±0.01
1	4.45±0.06	3.63±0.01	4.28±0.03	3.91±0.01
5	4.53±0.05	3.69±0.01	4.32±0.02	3.90±0.01
25	4.60±0.01	3.78±0.01	4.37±0.03	3.94±0.01
Control	4.37±0.01	3.62±0.05	4.29±0.06	3.84±0.04

*Arithmetic mean log₁₀ of cpm ± SE of quadruplicate cultures.

†Differences significantly different from controls at P<0.05.

cpm = counts per minute.

TABLE III Ability of immune cells to confer resistance to infection with *T pertenu* in irradiated, bone-marrow-reconstituted hamsters*

Recipients of:	Lesions sites	Treponemes $\times 10^4$ /node (mean \pm SE)	Lymph node weight (mg) (mean \pm SE)
Normal spleen cells			
Penicillin-treated	6/6	98 \pm 15	41 \pm 6
Non-penicillin-treated	6/6	103 \pm 24	30 \pm 5
Immune spleen cells			
Penicillin-treated	0/6	10 \pm 4†	16 \pm 2†
Non-penicillin-treated	0/6	8 \pm 3†	17 \pm 2†
Normal lymph node cells			
Penicillin-treated	6/6	64 \pm 4	44 \pm 4
Non-penicillin-treated	6/6	71 \pm 15	55 \pm 7
Immune lymph node cells			
Penicillin-treated	0/6	8 \pm 2†	21 \pm 2†
Non-penicillin-treated	0/6	12 \pm 3†	19 \pm 3

* Recipient hamsters were challenged with 1×10^6 *T pertenu*. Each hamster was inoculated at two sites. Data determined 21 days after infection.

† Differences significantly different from controls at $P < 0.05$.

framboesial hamsters are treated with a curative dose of penicillin to terminate the primary infection they are resistant to reinfection. Why treponemes persist at the primary foci of infection long after an effective immune defence has developed is unclear.

This persistence of *T pertenu* may be due in part to an induced immunodepression, as evidenced by diminished cellular responses. *T pertenu* exerts a profound effect on the ability of hamsters' lymphoid cells to respond to stimulation with mitogens. Throughout the course of the framboesial infection the lymph node cells responded poorly to con A, LPS, and PHA. This depression preceded the clinical signs of infection (lesions) and correlated well with the chronicity of framboesial infection. Treatment of framboesial hamsters with penicillin restored the mitogenic activity of the lymph node cells to be essentially equal or to slightly exceed that of controls. Likewise the variable responses of spleen cells to mitogenic stimulation did not differ from those of controls after the framboesial hamsters were treated with penicillin.

Antigenic (treponemal) overload may account in part for this impairment of framboesial lymphocytes. Since treatment with penicillin prevents proliferation of treponemes it reduces the amount of treponemal antigen and consequently the inhibition of normal responses to mitogens. As supporting evidence high concentrations of *T pertenu* antigen but not *T phagedenis* antigen impaired the mitogenic response and reduced the viability of normal lymphoid cells. In the absence of high concentrations of *T pertenu* antigen the spleen and lymph node cells responded modestly to mitogens.

It is not likely that impairment of lymphocyte stimulation was caused by serum factors, since the cells had multiple washes and we supplemented the lymphocyte cultures with different lots of FCS. In other treponemal studies impairment of lymphocyte responses has been shown with autologous and homologous serum.⁹⁻¹¹

Our results suggest that impairment is a property of the lymphocytes themselves. Impairment of mitogenic reactivity could be mediated by factors produced directly or indirectly by treponemes, since the lymph nodes of framboesial hamsters teem with treponemes and respond normally to mitogens after treatment with a curative dose of penicillin. These inhibiting factors may adhere avidly to lymphocytes and be dependent on the continued viability of the treponemes. Fitzgerald and Johnson^{12,13} have shown that a mucopolysaccharide, probably hyaluronic acid or chondroitin sulphate, accumulates within syphilitic chancres and on the surface of treponemes. Presentation of this material to peripheral blood lymphocytes from normal rabbits suppresses their response to con A.¹⁴ Another explanation may be that suppressor substances are produced by stimulated macrophages, T-cells, or macrophage-T-cell complexes.

Differences in the amount of treponemal antigen may also account for the ability of mitogenically unresponsive framboesial lymphocytes to elicit an effective immune response in recipients. No significant differences were detected among recipients of framboesial immune cells with or without mitogenic activity. Recipients of immune lymph node and spleen cells from penicillin-treated and non-penicillin-treated animals had no cutaneous lesions after 21 days of infection. They also had significantly lower lymph node weights and fewer treponemes per node than recipients of cells from penicillin-treated and non-penicillin-treated normal animals. In the initial infection accumulation of large amounts of treponemal antigen at the site of the primary infection may prevent lymphocytes from responding to treponemes and mitogens by blocking critical receptors of the cell membranes. Once the cells are removed from this paralysing environment, they can mobilise and elicit an effective treponemal immune response in normal recipients.

Since no correlation exists between the mitogenic activity of framboesial immune cells and their ability to elicit an effective immune response when transferred to non-immune recipients challenged with *T pertenu*, lymphocyte transformation in vitro is not a measure of effective treponemicidal activity. Our findings place in question the validity of this approach to assess the protective immune status of the framboesial host.

The immunological defence to framboesial infec-

tion is complex. In infected hamsters chronic skin lesions persist for six or seven months even in the presence of peak antitreponemal antibody titres.⁴ Throughout the course of infection lymph node cells respond poorly to stimulation with mitogens; but when the hamsters are cured with penicillin the mitogenic responses return to normal. Nine weeks after framboesial infection hamsters with or without lymphocyte mitogenic activity are resistant to reinfection. Cells from these hamsters confer protection on recipients and treatment of these cells with anti-thymocyte serum and complement abolishes their ability to transfer resistance.^{2,4} Yet treponemes persist at the primary foci of infection in the absence of antimicrobial treatment. These results suggest that hamsters develop the immune components necessary to resist reinfection but that these components are unable to destroy and eliminate the treponemes. Perhaps the amount of treponemal antigen or coating of treponemes with host protein or both prevents the developed effector mechanisms from functioning optimally.

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